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Use of Intravenous Infusion Study Design to Simultaneously Determine Brain Penetration and Systemic Pharmacokinetic Parameters in Rats[S]

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ABSTRACT

In drug discovery, the extent of brain penetration as measured by free brain/plasma concentration ratio ($K_{p,uu}$) is normally determined from one experiment after constant intravenous infusion, and pharmacokinetics (PK) parameters, including clearance (CL), volume of distribution at steady state (Vss), and effective half-life ($t_{1/2,eff}$) are determined from another experiment after a single intravenous bolus injection. The objective of the present study was to develop and verify a method to simultaneously determine $K_{p,uu}$ and PK parameters from a single intravenous infusion experiment. In this study, nine compounds (atenolol, loperamide, minoxidil, *N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine, sulpiride, and four proprietary compounds) were intravenously infused for 4 hours at 1 mg/kg or 24 hours at 1 or 6 mg/kg or bolus injected at 1 mg/kg. Plasma samples were serially collected, and brain and cerebrospinal fluid samples were collected at the end of infusion. The PK parameters were obtained using noncompartmental analysis (NCA) and compartmental analysis. The $K_{p,uu,brain}$ values of those compounds increased up to 2.86-fold from 4 to 24 hours. The CL calculated from infusion rate over steady-state concentration from the 24-hour infusion studies was more consistent with the CL from the intravenous bolus studies than that from 4-hour infusion studies (CL avg. fold of difference 1.19–1.44 vs. 2.10). The compartmental analysis using one- and two-compartment models demonstrated better performance than NCA regardless of study design. In addition, volume of distribution at steady state and $t_{1/2,eff}$ could be accurately obtained by one-compartment analysis within 2-fold difference. In conclusion, both unbound brain-to-plasma ratio and PK parameters can be successfully estimated from a 24-hour intravenous infusion study design.

SIGNIFICANCE STATEMENT

We demonstrated that the extent of brain penetration and pharmacokinetic parameters (such as clearance, V_{ss}, and effective $t_{1/2}$) can be determined from a single constant intravenous infusion study in rats.

Introduction

The blood-brain barrier (BBB) is a physiologic barrier formed by brain capillary endothelial cells with tight and adherens junctions (Rubin and Staddon, 1999; Abbott et al., 2010), which contribute to protecting the brain from endogenous and exogenous toxic compounds. In drug development, BBB is the major obstacle for the drug development targeting the central nervous system (CNS). The evaluation on the extent of brain penetration of drug candidates is one of the essential steps that is conducted during drug discovery and development for CNS diseases. It is also important for non–CNS-targeting drugs from a safety perspective. The brain penetration is commonly expressed as a brain-partitioning coefficient or brain-to-plasma concentration ratio based on either total and unbound concentrations at steady state [total brain-to-plasma ratio ($K_{p,brain}$) and unbound brain-to-plasma ratio ($K_{p,uu,brain}$)] (Hammarlund-Udenaes et al., 2009; Reichel, 2009; Freeman et al., 2019). Since only the protein unbound drug is assumed to bind to the target (Stain-Texier et al., 1999; Bouw et al., 2000) and produce therapeutic effects, the $K_{p,uu,brain}$ is more physiologically relevant and widely used to describe the extent of brain penetration. Moreover, it also provides insight into the transport mechanism of a compound at BBB (Bostrom et al., 2006; Chen et al., 2014; Summerfield et al., 2016). Because of these reasons, $K_{p,uu,brain}$ is an essential factor being considered with respect to pharmacology as well as pharmacokinetics of a compound in the early drug development (Hammarlund-Udenaes et al., 2008, 2009).

In general, the in vivo animal experiments to estimate $K_{p,uu,brain}$ and pharmacokinetics (PK) characteristics of a compound targeting CNS diseases are performed separately. For $K_{p,uu,brain}$ evaluation, 4-hour intravenous infusion study using a cassette dosing is commonly used to determine $K_{p,brain}$ values of several compounds in the discovery stage/step (Fridén et al., 2010; Nagaya et al., 2016). Then the PK characteristics of the ones with favorable brain penetration are further investigated via intravenous bolus injection. Although this study flow can accurately characterize the pharmacokinetics of a compound, it takes time and may not be cost-effective because two separate animal experiments are needed. If the key PK parameters of each compound can

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ABBREVIATIONS: AUC, area under the plasma concentration-time curve; BBB, blood-brain barrier; BSA, bovine serum albumin; CL, clearance; CNS, central nervous system; CSF, cerebrospinal fluid; $f_{u,brain}$, unbound fraction in the brain; $f_{u,p}$, unbound fraction in plasma; $K_{p,brain}$, total brain-to-plasma ratio; $K_{p,uu}$, free brain/plasma concentration ratio; $K_{p,uu,brain}$, unbound brain-to-plasma ratio; $K_{p,uu,CSF}$, unbound CSF-to-plasma ratio; NCA, noncompartmental analysis; NFPS, *N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine; PK, pharmacokinetics; $t_{1/2,terminal}$, terminal half-life; $t_{1/2,eff}$, effective half-life; V_{ss}, volume of distribution at steady state.

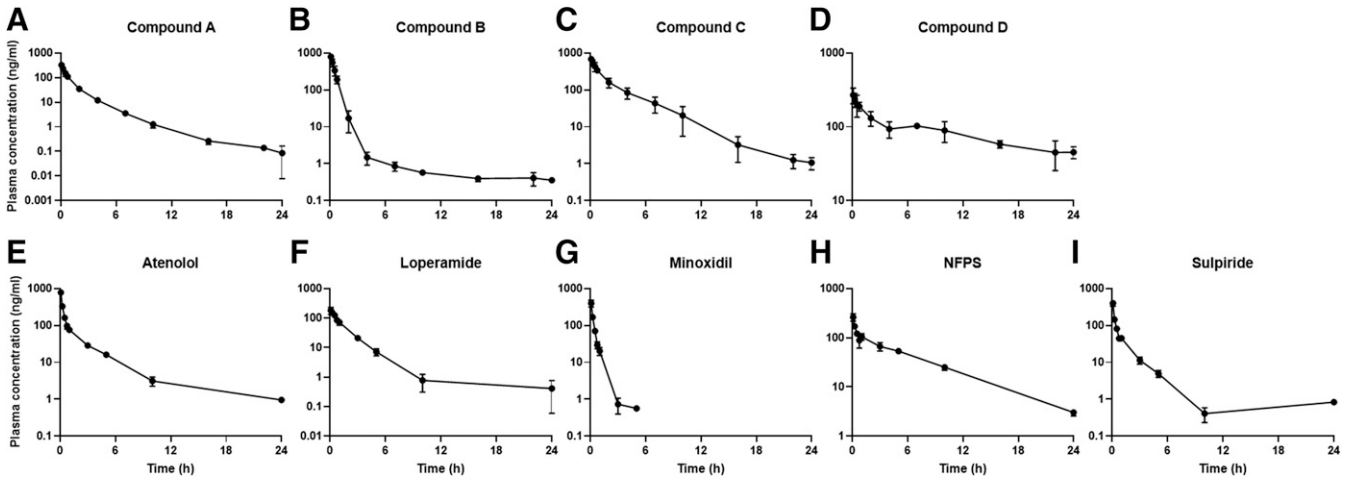


Fig. 1. Plasma concentration vs. time profiles of (A–D) internal and (E–I) commercial compounds in rats after single intravenous injection as a cassette dosing (1 mg/kg). The closed circles represent observed data (n = 3; mean ± S.D.).

be simultaneously estimated along with $K_{p,uu,brain}$ from the same study, we can improve the efficiency for the experiment and reduce the usage of animals.

Herein, we performed intravenous bolus and intravenous infusion studies for 4 or 24 hours to evaluate $K_{p,uu,brain}$ of nine compounds in rats, among which five were commercially available compounds, and four were proprietary compounds. Those compounds represent compounds with a wide range of clearance (CL) (i.e., low, medium, and high). One- and two-compartment models were applied to estimate the PK parameters [e.g., CL, V_{ss}, and effective half-life ($t_{1/2,eff}$)], and the performance of the two models was evaluated as compared with the PK parameters after intravenous bolus injection.

Materials and Methods

Chemicals. Atenolol, loperamide, minoxidil, and sulpiride were obtained from Sigma-Aldrich (St. Louis, MO), and *N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl]sarcosine (NFPS) was purchased from Tocris Bioscience (Minneapolis, MN). Four proprietary compounds (compounds A, B, C, and D) were synthesized at Biogen. All chemicals used in the experiments were of the highest available grade.

Animal Experiments. Jugular vein and carotid artery cannulated male Sprague-Dawley rats were purchased from Charles River Laboratory (Wilmington, MA). Upon arrival, the rats were acclimated for at least 3 days on a 12-hour light/dark cycle in a temperature- and humidity-controlled environment with free access to food and water. Animal experiments for proprietary and commercial compounds were separately conducted as two cassette administrations to rats, respectively (Nagilla et al., 2011; Liu et al., 2012). For both proprietary and commercial compounds, the dosing solution was prepared by dissolving compounds with 20% captisol and filtered with 0.2-μm syringe filter (Pall Life Sciences, Port Washington, NY). Then compounds were intravenously injected (1 mg/kg) or infused over 4 hours (1 mg/kg) or 24 hours (1 and 6 mg/kg) in rats (n = 3 for each group). Blood samples (100 μl) were serially collected from the left carotid artery into EDTA-containing tubes (SAI Infusion Technologies, Lake Villa, IL) at predetermined time points after dosing. For proprietary compounds, blood samples were collected prior to dosing and at 0.083, 025, 0.5, 0.75, 2, 4 (last sampling for 4-hour infusion group), 7, 10, 16, 22, and 24 hours postdosing. For commercial compounds, blood samples were withdrawn prior to dosing and at 0.083, 0.25, 0.5, 0.75, 1, 3, 5, 10, and 24 hours after a single intravenous injection and at 0.5, 1, 2, 4 (last sampling for 4-hour infusion group), 8, 16, 22, and 24 hours after intravenous infusion. After blood sampling, the same volume of heparinized saline (20 IU/ml) was injected to compensate for blood loss in rats. Plasma samples were prepared after centrifugation at 10,000 rpm for 5 minutes. At the last sampling time, cerebrospinal fluid (CSF) samples were collected from cisterna magna after CO_2 euthanasia and were immediately diluted with the equivalent volume of 8% bovine serum albumin (BSA) in PBS. Then, brain samples were harvested. The collected samples were stored at −80°C until analysis. All animal experiments were approved by the Institutional Animal Care and Use Committee at Biogen, and the study was conducted in compliance with the institutional guidelines.

Sample Analysis. Standard calibration curves were prepared using a serial dilution scheme of analytes in blank rat matrix. All standard calibrants were aliquoted into the extraction plate and normalized at a ratio of 1:1:1 to contain an equal mixture of plasma, brain homogenate, and artificial cerebrospinal fluid and 8% BSA (5:5 v/v). The collected brain samples were homogenized with two times of volume (w/v) of PBS (pH 7.4), and 20 μl brain homogenate was mixed with the same volume of blank plasma and mixture of artificial cerebrospinal fluid and 8% BSA (5:5 v/v). For plasma sample preparation, 20 μl of plasma sample was mixed with the same volume of brain homogenate and blank mixture of artificial cerebrospinal fluid and 8% BSA (5:5 v/v). For CSF sample preparation, 20 μl of CSF sample was added into 20 μl of each blank plasma and brain homogenate. Then proteins in a total of 60 μl of the standard, matrix blanks, or a sample were precipitated with 360 μl of acetonitrile or acetonitrile containing internal standards (glyburide, carbutamide, and chrysin). After vortexing and centrifuging at 3500 rpm for 10 minutes, 250 μl of supernatant was transferred into a 96-well injection plate and dried under nitrogen gas at 40° C. Then samples were reconstituted with 100 μl the mixture of water and acetonitrile (50:50 v/v) and analyzed with high-performance liquid chromatography equipped with mass spectrometry (Triple Quad 5500 System; AB Sciex, Framingham, MA). Mobile phases used were 0.1% formic acid in water and 0.1% formic acid in acetonitrile along with an Ace EXCEL 3 C18-PFP 2.1 × 50–mm column (3 μm particle size; Advanced Chromatography Technologies Ltd., Aberdeen, Scotland).

Single Intravenous Bolus Injection Data Analysis. Noncompartmental analysis (NCA) was applied to estimate PK parameters from the intravenous bolus injection data. PK parameters including CL, volume of distribution at steady state (V_{ss}), dose-normalized area under the plasma concentration-time curve (AUC_∞/dose), and terminal half-life ($t_{1/2,terminal}$) were estimated by Phoenix WinNonlin (version 7.0; Pharsight Corporation, Cary, NC). The $t_{1/2,eff}$ was calculated using eq. 1. It was proposed to reflect drug accumulation after multiple doses (Boxenbaum and Battle, 1995), whereas $t_{1/2,terminal}$ is a dependent parameter upon elimination phase. This treatment is a simplification of a more complex pharmacokinetic process in drug discovery wherein $t_{1/2}$ is estimated from predicted CL and V_{ss}.

$$t_{1/2,eff} = \frac{\ln 2 \times V_{ss}}{CL} \quad (1)$$

TABLE 1

Pharmacokinetic parameters calculated by NCA after 1 mg/kg single intravenous injection of compounds (n = 3 for each group)

Data were shown as mean ± S.D.

	Internal Compounds				Commercial Compounds				
	A	B	C	D	Atenolol	Loperamide	Minoxidil	NFPS	Sulpiride
CL (ml/min/kg)	28.0 ± 3.28	31.3 ± 7.95	7.44 ± 2.07	3.29 ± 0.91	35.5 ± 2.56	22.5 ± 4.24	113 ± 17.2	18.4 ± 2.05	82.9 ± 8.86
V_{ss} (l/kg)	2.80 ± 0.30	11.5 ± 15.5	1.42 ± 0.25	3.99 ± 0.94	5.56 ± 1.03	2.72 ± 0.47	6.96 ± 6.93	7.07 ± 0.69	8.25 ± 2.31
AUC_{inf}/dose (ng·h/ml/mg/kg)	600 ± 69.2	560 ± 166	2350 ± 580	5330 ± 1430	471 ± 32.7	757 ± 131	149 ± 23.1	912 ± 95.6	204 ± 22.5
$t_{1/2,terminal}$ (h)	3.46 ± 1.73	48.9 ± 72.0	6.80 ± 6.58	14.9 ± 8.65	3.71 ± 1.53	2.14 ± 0.78	4.53 ± 7.15	4.59 ± 0.22	1.89 ± 1.18
$t_{1/2,eff}$ (h)	1.16 ± 0.06	5.65 ± 8.38	2.25 ± 0.30	15.1 ± 7.07	1.83 ± 0.45	1.43 ± 0.41	0.79 ± 0.88	4.44 ± 0.13	1.18 ± 0.45
$f_{u,p}$	0.00375[a]	0.135[a]	0.0131[a]	0.0266[a]	0.91[b]	0.0701[c]	0.640[d]	0.041[e]	0.880[c]
$f_{u,brain}$	0.00613[a]	0.0592[a]	0.0106[a]	0.00402[a]	0.599[d]	0.00196[c]	0.658[d]	0.0017[e]	0.345[c]
$f_{u,csf}$[f]	1.0	0.9995	1.0	0.9999	0.9706	0.9998	0.9947	0.9999	0.9785

[a]Data were obtained from the internal data base.
[b]Obtained from Srikanth et al. (2013).
[c]Obtained from Kodaira et al. (2011).
[d]Obtained from Liu et al. (2018). For minoxidil, it was assumed that unbound fractions in blood and plasma are the same.
[e]Obtained from Liu et al. (2005).
[f]Calculated by eq. 5 (Fridén et al., 2009).

Intravenous Infusion Data Analysis. Total ($K_{p,brain}$) and unbound ($K_{p,uu,brain}$) brain-to-plasma partition coefficients as well as the unbound CSF-to-plasma ratio ($K_{p,uu,CSF}$) were calculated as follows:

$$K_{p,brain} = \frac{C_{brain}}{C_p} \quad (2)$$

$$K_{p,uu,brain} = \frac{C_{brain} \cdot f_{u,brain}}{C_p \cdot f_{u,p}} \quad (3)$$

$$K_{p,uu,CSF} = \frac{C_{CSF} \cdot f_{u,CSF}}{C_p \cdot f_{u,p}} \quad (4)$$

C_{brain}, C_p, and C_{CSF} are total brain, plasma, and CSF concentrations at the end of infusion, respectively, and $f_{u,brain}$ and $f_{u,p}$ are unbound fractions in the brain and plasma, respectively. The unbound fraction in the CSF ($f_{u,CSF}$) was calculated from $f_{u,p}$ using a single binding site model as follows (Fridén et al., 2009):

$$f_{u,CSF} = \frac{1}{1 + Q_{alb}\left(\frac{1}{1 - f_{u,p}} - 1\right)} \quad (5)$$

Q_{alb} is the ratio of albumin in CSF over that in plasma, which was set to 0.003 for rats (Habgood et al., 1992).

Noncompartmental analysis was applied to determine CL from 4- to 24-hour infusion data using the following equation:

$$CL = \frac{\text{Infusion rate}}{C_{ss}} \quad (6)$$

The plasma concentration at steady state (C_{ss}) was defined as the plasma concentration at 4 hours for the 4-hour infusion study and average plasma concentration of 22 and 24 hours for the 24-hour infusion study. In addition, the compartmental analysis was performed using both one- and two-compartment models, which were incorporated in Phoenix WinNonlin (version 7.0; Pharsight Corporation), to estimate the PK parameters, particularly for V_{ss}, which cannot be obtained via noncompartmental analysis in this study. Using the obtained CL and V_{ss}, the $t_{1/2,eff}$ was calculated using eq. 1 (Gunaydin et al., 2018; Smith et al., 2018), since the $t_{1/2,terminal}$ could not be estimated because of the lack of elimination phase of the infusion data.

The PK parameters were shown as mean ± S.D. The one-way ANOVA with post hoc Tukey's test was performed to compare $K_{p,brain}$ or $K_{p,uu,brain}$ values among different study designs using GraphPad Prism (version 8.3.0; San Diego, CA), and P values <0.05 were considered statistically significant. Moreover, the estimated CL, V_{ss}, and $t_{1/2,eff}$ values of each compound from constant infusion studies were divided by those from bolus injection to obtain the fold difference to compare the performance of different study designs as well as the data analysis methods. The average fold difference was calculated to assess the performance of different estimation approaches. Simple linear regression was performed to seek correlations of the calculated PK parameters after a single intravenous injection with the estimated PK parameters by NCA and/or compartmental analyses after

TABLE 2

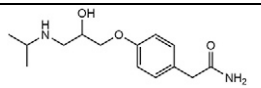
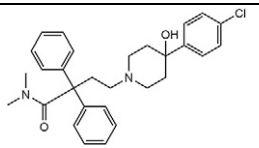
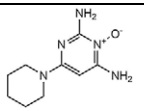
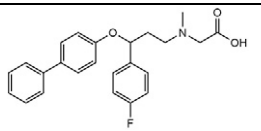
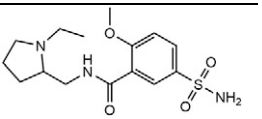
Calculated total ($K_{p,brain}$) and unbound ($K_{p,uu,brain}$) brain- and unbound CSF ($K_{p,uu,CSF}$)-to-plasma ratios

	Internal Compounds				Commercial Compounds				
	A	B	C	D	Atenolol	Loperamide	Minoxidil	NFPS	Sulpiride
$K_{p,brain}$									
1 mg/kg over 4 h	0.49 ± 0.14	0.23 ± 0.06	0.30 ± 0.05	3.11 ± 0.44	0.08 ± 0.01	0.15 ± 0.03	0.14 ± 0.03	0.33 ± 0.04	0.11 ± 0.01
1 mg/kg over 24 h	0.72 ± 0.12	0.21 ± 0.07	0.47 ± 0.05	4.04 ± 0.68	0.09 ± 0.01	0.10 ± 0.02	0.29 ± 0.17	1.01 ± 0.38*	0.18 ± 0.01
6 mg/kg over 24 h	0.81 ± 0.14	0.28 ± 0.06	0.51 ± 0.10*	3.73 ± 0.54	0.09 ± 0.02	0.15 ± 0.04	0.19 ± 0.08	0.74 ± 0.18	0.19 ± 0.05*
$K_{p,uu,brain}$[a]									
1 mg/kg over 4 h	0.80 ± 0.23	0.10 ± 0.03	0.24 ± 0.04	0.47 ± 0.07	0.05 ± 0.01	0.004 ± 0.001	0.14 ± 0.03	0.014 ± 0.001	0.044 ± 0.003
1 mg/kg over 24 h	1.17 ± 0.20	0.09 ± 0.03	0.38 ± 0.04	0.61 ± 0.10	0.06 ± 0.004	0.003 ± 0.001	0.29 ± 0.17	0.04 ± 0.02*	0.072 ± 0.005*
6 mg/kg over 24 h	1.32 ± 0.23	0.12 ± 0.03	0.41 ± 0.08*	0.56 ± 0.08	0.06 ± 0.01	0.004 ± 0.001	0.20 ± 0.09	0.03 ± 0.01	0.076 ± 0.021*
$K_{p,uu,CSF}$[b]									
1 mg/kg over 4 h	N.A.	N.A.	N.A.	N.A.	0.094 ± 0.044	0.318 ± 0.143	0.14 ± 0.02	0.253 ± 0.088	0.099 ± 0.001
1 mg/kg over 24 h	N.A.	N.A.	N.A.	N.A.	0.091 ± 0.058	0.451[c]	0.17[c]	0.412[c]	0.13 ± 0.07
6 mg/kg over 24 h	N.A.	N.A.	N.A.	N.A.	0.081 ± 0.018	0.126[c]	0.16 ± 0.05	0.169 ± 0.057	0.12 ± 0.01

N.A., not available because CSF samples were not collected; N.C., not calculated because drug conc. in CSF was not detectable.
[a]Unbound brain-to-plasma ratio was calculated using eq. 3, and unbound fractions in plasma and brain were shown in Table 1.
[b]CSF-to-unbound plasma ratio was calculated using eq. 4, and unbound fractions in plasma and CSF were shown in Table 1.
[c]S.D. was not available, since the sample size was fewer than 3.
*$P < 0.05$ compared with 1 mg/kg infusion over 4 h.

TABLE 3

Physicochemical and PK properties of test compounds from internal data base and literature

	Internal Compounds				Commercial Compounds				
	A	B	C	D	Atenolol	Loperamide	Minoxidil	NFPS	Sulpiride
Structure	N.A.	N.A.	N.A.	N.A.					
Physicochemical properties									
LogP[a]	6.28	1.38	5.7	2.89	0.5	5.15	1.24	4.83	0.43
LogS[b]	−6.66	−3.25	−6.14	−4.96	−1.50	−6.49	−2.84	−5.92	−2.81
PK properties (in vivo)									
Hepatic extraction ratio (E_H)	0.707[c]	0.349[c]	0.088[c]	0.037[c]	0.3[d]	N.A.	N.A.	N.A.	N.A.
CL (ml/min/kg)	N.A.	N.A.	N.A.	N.A.	38.9[e] 24.5[f]	128[g]	N.A.	13[h]	41.0[i]
V_{ss} (l/kg)	N.A.	N.A.	N.A.	N.A.	7.02[e] 2.88[f]	9.0[g]	N.A.	N.A.	2.11[i]
AUC_{inf}/dose (ng·h/ml/mg/kg)	N.A.	N.A.	N.A.	N.A.	789[j] 765[k]	131[g]	N.A.	N.A.	402[i]
$t_{1/2,terminal}$ (h)	N.A.	N.A.	N.A.	N.A.	2.21[e] 2.76[f]	1.0[g]	N.A.	3.3[h]	0.94[i]
$K_{p,uu,brain}$	N.A.	N.A.	N.A.	N.A.	0.026[l] 0.034[m]	0.00886[n]	0.175[m]	0.018[o] 0.12[o]	0.0219[n]
$K_{p,uu,CSF}$	N.A.	N.A.	N.A.	N.A.	0.036[l]	0.0376[n]	N.A.	0.13[o]	0.0499[n]
PK properties (in vitro)									
Efflux ratio (MDCK-MDR1)	2.16[p]	5.68[p]	1.40[p]	0.865[p]	0.416[f] 0.69[q] 2.33[r]	17.8[r] 212[s]	1.3[s] 3[t]	N.A.	0.9[u]
Efflux ratio (MDCK-BCRP)	1.30[p]	21.0[p]	0.548[p]	0.46[p]	1.43[q]	27.0[p]	4.2[p]	N.A.	0.76[n]

BCRP, breast cancer resistance protein; MDCK, Madin-Darby Canine Kidney; MDR1, multidrug resistance gene; N.A., not available.

[a]LogP values for internal and commercial compounds except for minoxidil were calculated by using ChemDraw Professional 16 (PerkinElmer Informatics, Inc). LogP value for minoxidil was obtained from PubChem.

[b]LogS values for internal and commercial compounds were calculated by using ChemDraw Professional 16 (PerkinElmer Informatics, Inc).

[c]Hepatic extraction ratio or E_H for internal compounds was calculated by hepatic clearance (CL_H)/hepatic blood flow (Q_H). The Q_H was set to 55 ml/min per kilogram (Davies and Morris, 1993), and CL_H was obtained from rat hepatocytes stability test of internal data base.

[d]Data were obtained from Hung et al. (2001).

[e]Data were obtained from Belpaire et al. (1990).

[f]Data were obtained from Chen et al. (2020).

[g]Data were obtained from Zamek-Gliszczynski et al. (2012).

[h]Data were obtained from Liu et al. (2005) after subcutaneous injection. The bioavailability was assumed to be 100%.

[i]Data were obtained from Yamada et al. (1990).

[j]Data were obtained from Lemmer et al. (1985).

[k]Data were obtained from Mehvar et al. (1990).

[l]Data were obtained from Fridén et al. (2009).

[m]Data were obtained from Liu et al. (2018).

[n]Data were obtained from Kodaira et al. (2011). The efflux ratio of sulpiride was obtained by using mouse Bcrp-transfected MDCK.

[o]Data were obtained from Liu et al. (2006).

[p]Data were obtained from the internal data base.

[q]Data were obtained from Bicker et al. (2017).

[r]Data were obtained from Hellinger et al. (2012).

[s]Data were obtained from Li et al. (2013).

[t]Data were obtained from Nagar et al. (2014).

[u]Data were obtained from Feng et al. (2008).

TABLE 4

CL values estimated by noncompartmental and compartmental analyses based on 4- and 24-h intravenous infusion studies

Each value was normalized to the CL of intravenous bolus data and then shown as avg. fold difference. The actual mean CL values for each group were shown in the bracket (milliliter per minute per kilogram).

	Internal Compounds				Commercial Compounds					Total	
	A	B	C	D	Atenolol	Loperamide	Minoxidil	NFPS	Sulpiride	Avg.	CV%
NCA											
Intravenous bolus	1 (28.0)	1 (31.3)	1 (7.44)	1 (3.29)	1 (35.5)	1 (22.5)	1 (113.4)	1 (18.4)	1 (82.9)	1	0
NCA											
1 mg/kg over 4 h	0.81 (22.6)	1.12 (35.1)	1.22 (9.10)	4.48 (14.7)	1.61 (57.3)	4.55 (103)	0.99 (113)	2.78 (51.1)	1.30 (108)	2.10	70.8
1 mg/kg over 24 h	0.71 (19.9)	2.49 (77.9)	0.76 (5.66)	1.40 (4.62)	1.27 (45.0)	3.10 (69.9)	0.90 (101.6)	1.22 (22.4)	1.15 (95.0)	1.44	56.4
6 mg/kg over 24 h	0.85 (23.9)	2.05 (64.1)	1.02 (7.61)	1.26 (4.14)	0.65 (23.0)	3.03 (68.3)	0.62 (70.4)	0.66 (12.1)	0.62 (51.1)	1.19	69.3
One-compartment analysis											
1 mg/kg over 4 h	0.57 (16.1)	0.99 (31.0)	0.61 (4.58)	3.57 (11.7)	1.66 (59.0)	4.07 (91.8)	1.02 (116)	2.56 (47.1)	1.29 (107)	1.81	71.0
1 mg/kg over 24 h	0.73 (20.5)	2.29 (71.7)	0.83 (6.20)	1.21 (3.98)	1.23 (43.7)	3.29 (74.2)	0.90 (102.5)	1.16 (21.4)	1.11 (91.7)	1.42	58.9
6 mg/kg over 24 h	0.89 (25.0)	1.42 (44.4)	1.01 (7.54)	0.86 (2.84)	0.68 (24.0)	2.91 (65.6)	0.61 (68.8)	0.61 (11.1)	0.62 (51.6)	1.07	69.2
Two-compartment analysis											
1 mg/kg over 4 h	0.57 (16.0)	0.99 (31.0)	0.61 (4.54)	3.92 (12.9)	1.53 (54.3)	4.07 (91.8)	0.89 (101)	2.55 (47.0)	1.27 (106)	1.82	75.1
1 mg/kg over 24 h	0.70 (19.7)	2.14 (66.9)	0.62 (4.60)	0.87 (2.88)	1.23 (43.6)	2.87 (64.6)	0.90 (102.4)	1.16 (21.3)	1.11 (91.7)	1.29	57.4
6 mg/kg over 24 h	0.83 (23.2)	1.42 (44.4)	0.98 (7.30)	0.64 (2.09)	0.66 (23.4)	3.85 (86.9)	0.60 (68.1)	0.60 (11.1)	0.61 (50.8)	1.13	93.1

intravenous infusion. The correlations plots for CL, V_{ss}, and $t_{1/2,eff}$ were depicted in Supplemental Figs. 1–3, respectively.

Results

Pharmacokinetics after a Single Intravenous Injection. The plasma-concentrations-versus-time profiles of the proprietary (four compounds) and commercial compounds (five compounds) after the single intravenous injection were depicted in Fig. 1, and the estimated PK parameters for each compound were shown in Table 1. Most of the compounds exhibited lower CL than the hepatic blood flow rate (55 ml/min/kg) (Davies and Morris, 1993) except for minoxidil (113 ml/min/kg) and sulpiride (82.9 ml/min/kg). For V_{ss}, all of the test compounds were higher than 1 l/kg (Kwon, 2001), although some had large variations in V_{ss} possibly due to a small sample size. The $t_{1/2,terminal}$ ranged between 1.89 and 48.9 hours, whereas the $t_{1/2,eff}$ values were relatively smaller than $t_{1/2,terminal}$, for which the values were between 0.79 and 15.1 hours (Table 1). The fractions of the unbound plasma and brain protein binding ($f_{u,p}$ and $f_{u,brain}$) for each compound were collected from the literature or generated in house (Table 1) (Liu et al., 2005, 2018; Kodaira et al., 2011; Srikanth et al., 2013). Five and six of the nine compounds were highly bound to proteins in plasma and brain ($f_{u,p}$ and $f_{u,brain} < 0.1$), respectively. Among the compounds with relatively low protein binding, the $f_{u,p}$ and $f_{u,brain}$ values were 0.135 and 0.0592 for compound B, 0.640 and 0.658 for minoxidil, and 0.880 and 0.345 for sulpiride, respectively, and atenolol showed the lowest protein binding among the tested compounds in both plasma and brain (Table 1). The $f_{u,CSF}$ values for all of the test compounds calculated by eq. 5 were higher than 0.9706, indicating most of compounds in CSF presented as unbound forms.

Brain Penetration of the Compounds: $K_{p,uu,brain}$ at 4 hours Is Lower than $K_{p,uu,brain}$ at 24 hours for Most Compounds. The $K_{p,brain}$, $K_{p,uu,brain}$, and $K_{p,uu,CSF}$ values for each compound were calculated using eqs. 2–4, respectively (Table 2). Overall, both $K_{p,brain}$ and $K_{p,uu,brain}$ values after 4-hour intravenous infusion were lower compared with the values in the 24-hour intravenous infusion studies except for those of compound B and loperamide, whose $K_{p,brain}$ and $K_{p,uu,brain}$ values were similar in both 4- and 24-hour infusion studies. In particular, notable increases in $K_{p,brain}$ and $K_{p,uu,brain}$ values of compound C, NFPS, and sulpiride were observed by prolonged infusion time from 4 to 24 hours (Table 2). Despite the same infusion rate of 1 mg/kg/4 h and 6 mg/kg/24 h infusion studies, the calculated $K_{p,brain}$ and $K_{p,uu,brain}$ for compound C, NFPS, and sulpiride in 24-hour infusion groups were 70%, 224%, and 73% higher than those in 4-hour infusion groups, respectively. Interestingly, the compounds with a longer half-life (>4 hours), such as NFPS, exhibited a significant increase in brain penetration ($K_{p,uu,brain}$) by prolonged infusion time. $K_{p,brain}$ and $K_{p,uu,brain}$ values of the compounds A, C, and D were slightly increased by 1.20–1.70-fold after 24-hour infusion, and there were no differences in $K_{p,brain}$ and $K_{p,uu,brain}$ of compound B and atenolol between 4- and 24-hour infusion studies.

The CSF samples were only collected from the studies with the commercial compounds. Some of the determined CSF concentrations of loperamide, minoxidil, and NFPS were below the limit of quantification. Therefore, $K_{p,uu,CSF}$ for those compounds could not be calculated or was shown without S.D. (n = 1–2) (Table 2). The average fold differences between $K_{p,uu,CSF}$ and $K_{p,uu,brain}$ for atenolol, minoxidil, and sulpiride were about within 2 folds, whereas loperamide and NFPS showed substantial differences between $K_{p,uu,CSF}$ and $K_{p,uu,brain}$. The observed $K_{p,uu,brain}$ and $K_{p,uu,CSF}$ of commercial compounds were comparable with the reported values in the literature except $K_{p,uu,CSF}$ of loperamide (Table 3).

CL from Intravenous Infusion Studies: 24-Hour Infusion Studies Provided More Accurate CL than the 4-Hour Infusion Studies. The CL values were calculated using NCA with equation CL = infusion rate/C_{ss} (eq. 6), and PK parameters including CL, V_{ss}, and $t_{1/2,eff}$ were estimated using compartmental analyses and then normalized to the PK parameters determined from intravenous injection data for each compound (Tables 4–6). The observed data and the fitted results by one- and two-compartment models based on infusion data were depicted in Figs. 2–4. Overall, most of the estimated CL from the constant intravenous infusion data by both noncompartmental and compartmental analyses were within 2-fold difference compared with those of the single intravenous bolus data, whereas NCA method based on 4-hour infusion data slightly overestimated CL by more than 2 folds (Table 4). The average fold difference of CL in the 24-hour infusion groups normalized to the CL data of the intravenous bolus groups was much closer to 1 than that in the 4-hour infusion groups regardless of noncompartmental and compartmental analyses (Table 4). The CL tends to be overestimated in the 4-hour infusion studies probably due to not having enough time for these compounds to reach the steady states in vivo. The average fold differences of the CL values determined by one- and two-compartment models in the 24-hour infusion studies ranged between 1.07–1.42 and 1.13–1.29, respectively, whereas the average fold differences of the CL

values determined by both models in the 4-hour infusion studies were between 1.81 and 1.82, suggesting that the 24-hour infusion studies provided more accurate CL than the 4-hour infusion studies (Table 4). In particular, the calculated CL of compound D in the 4-hour infusion study by NCA was 4.48-fold higher than the actual CL obtained after single intravenous bolus injection, whereas the average fold differences were less than 1.40 in the 24-hour infusion studies (estimated by the same NCA approach) (Table 4).

With respect to the methodology for CL estimation, the average fold differences of CL estimated by NCA and one- and two-compartment models were 2.10, 1.81, and 1.82 for 1 mg/kg/4 h study; 1.44, 1.42, and 1.29 for 1 mg/kg/24 h study; and 1.19, 1.07, and 1.13 for 6 mg/kg/24 h study, respectively (Table 4), indicating that NCA provided the most inaccurate CL among the estimation methods (NCA, one- and two-compartmental analyses) regardless of the study design. In comparison, the observed data were well described by both one- and two-compartment models (Figs. 2–4), and the estimated CL values by both models were similar and more accurate than the values by NCA. The average fold differences of the CL values determined by one- and two-compartment models in the 4- and 24-hour infusion studies ranged between 1.07–1.81 and 1.13–1.82, respectively, whereas the average fold differences of the CL values determined by NCA in the 4- and 24-hour infusion studies were between 1.19 and 2.10, suggesting that the compartmental analyses provided more accurate CL than NCA (Table 4).

V_{ss} from Intravenous Infusion Study: the 24-Hour Infusion Studies Provided More Accurate V_{ss} than 4-Hour Infusion Studies. The compartmental analyses using one- and two-compartment models were applied to estimate V_{ss} from the 4- and 24-hour infusion studies. The average fold differences of the V_{ss} values were within 2 folds between the 4- and 24-hour infusion studies except for the estimated V_{ss} by two-compartment model based on the 1 mg/kg/24 h infusion studies (Table 5). The estimated V_{ss} from the 4-hour infusion studies tended to be underestimated by both one- and two-compartment models compared with that in the intravenous bolus studies; the average fold differences for one- and two-compartment models were 0.58 and 0.79, respectively. Interestingly, the 24-hour infusion studies provided more accurate V_{ss} than 4-hour infusion studies.

Application of Compartmental Analyses for $t_{1/2,eff}$ Estimation from Intravenous Infusion Study without Elimination Phase Showed the Average Fold Differences Were Close to 1 Fold or within 2 Folds of the Actual Values. The $t_{1/2,eff}$ was indirectly derived from the estimated CL and V_{ss} using eq. 1. Therefore, only the values from the compartmental analyses using one- and two-compartment models were used to derive $t_{1/2,eff}$ (Table 6). Among the tested compounds, the $t_{1/2,eff}$ of compound D was poorly predicted in the 4-hour infusion study because the calculated $t_{1/2,eff}$ values by one- and two-compartment models were 15% and 11% of the actual values. For most of other compounds, the average fold differences in the $t_{1/2,eff}$ were very close to 1 fold or within 2 folds of the actual value (Table 6). Overall, using CL and V_{ss} estimated by one-compartment model showed slightly better performance in predicting $t_{1/2,eff}$ than using the estimates by two-compartment model (Table 6).

TABLE 5

V_{ss} estimated by one- and two-compartment models based on 4- and 24-h intravenous infusion studies

Each value was normalized to the V_{ss} of intravenous bolus data and then shown as avg. fold difference. The actual mean V_{ss} values for each group were shown in the bracket (liter per kilogram).

	Internal Compounds				Commercial Compounds					Total (with Loperamide)		Total (without Loperamide)	
	A	B	C	D	Atenolol	Loperamide	Minoxidil	NFPS	Sulpiride	Avg.	CV%	Avg.	CV%
NCA													
Intravenous bolus	1 (2.80)	1 (11.5)	1 (1.42)	1 (3.99)	1 (5.56)	1 (2.72)	1 (6.96)	1 (7.07)	1 (8.25)	1	0	1	0
One-compartment analysis													
1 mg/kg over 4 h	0.99 (2.77)	0.20 (2.29)	1.02 (1.45)	0.50 (2.01)	0.39 (2.14)	0.76 (2.06)	0.35 (2.45)	0.63 (4.43)	0.38 (3.11)	0.58	50.2	0.56	54.4
1 mg/kg over 24 h	1.60 (4.48)	0.45 (5.22)	0.90 (1.29)	0.70 (2.81)	0.61 (3.40)	8.41 (22.9)	0.60 (4.19)	1.04 (7.35)	0.62 (5.13)	1.66	154	0.82	44.9
6 mg/kg over 24 h	1.13 (3.18)	0.17 (1.92)	1.32 (1.87)	0.83 (3.32)	0.68 (3.80)	8.07 (22.0)	0.67 (4.66)	0.72 (5.12)	0.75 (6.19)	1.59	154	0.79	43.6
Two-compartment analysis													
1 mg/kg over 4 h	0.99 (2.77)	0.57 (6.51)	1.24 (1.77)	0.34 (1.34)	0.65 (3.63)	0.76 (2.06)	1.27 (8.87)	0.74 (5.23)	0.52 (4.30)	0.79	41.0	0.79	43.6
1 mg/kg over 24 h	1.24 (3.48)	0.83 (9.49)	1.98 (2.81)	0.96 (3.82)	0.61 (3.41)	13.7 (37.3)	1.17 (8.16)	1.09 (7.74)	0.91 (7.50)	2.50	169	1.10	37.1
6 mg/kg over 24 h	1.02 (2.87)	0.60 (6.85)	1.71 (2.42)	1.30 (5.18)	0.83 (4.62)	2.86 (7.79)	0.73 (5.10)	0.73 (5.13)	0.86 (7.07)	1.18	60.7	0.97	37.7

Discussion

Brain penetration of compounds is a highly essential element in the drug discovery stage for CNS diseases. Thus, animal experiments should be adequately designed to accurately determine $K_{p,uu,brain}$ of the tested compounds with various and different physicochemical properties. Although a cassette dosing with intravenous infusion for 4 hours is typically conducted to determine $K_{p,uu,brain}$ in rats (Fridén et al., 2010;

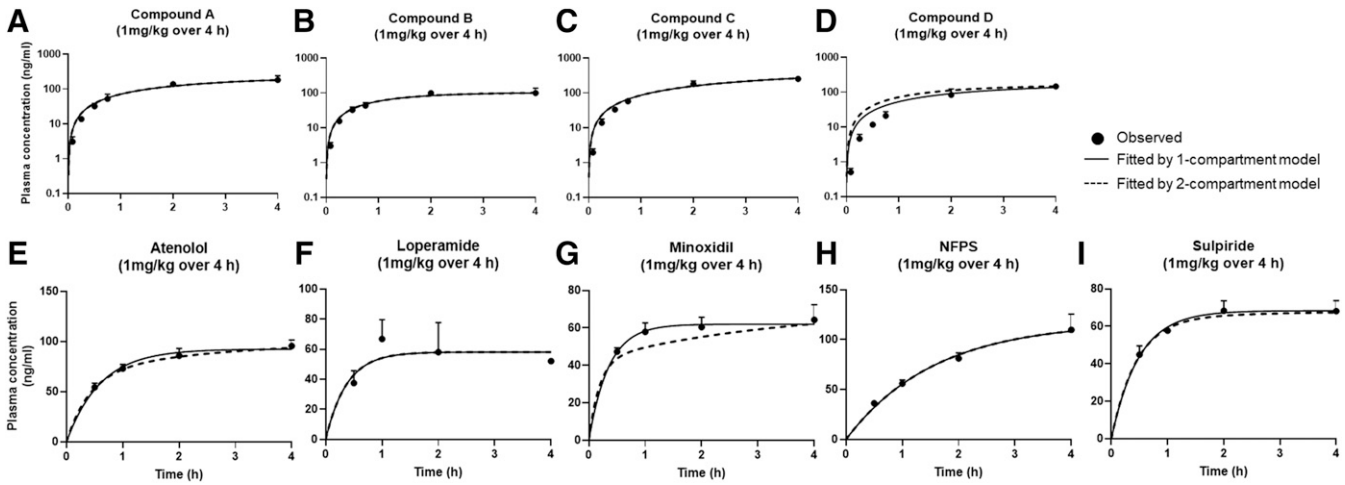


Fig. 2. Plasma concentration vs. time profiles of (A–D) internal and (E–I) commercial compounds in rats after a constant intravenous infusion over 4 hours as a cassette dosing (1 mg/kg). The closed circles represent observed data (n = 3; mean ± S.D.), and the fitted results by one- and two-compartment models are depicted as solid and dashed lines, respectively.

Nagaya et al., 2016), 4-hour infusion may not be long enough to reach the steady state for $K_{p,uu,brain}$ evaluation if the compound has a longer half-life (Zheng, 2015). Thus, the longer duration of infusion is necessary for the compounds to reach equilibrium in plasma and brain, as demonstrated by the current study.

Comparing $K_{p,brain}$ and $K_{p,uu,brain}$ values from the 4- and 24-hour infusion studies with the same infusion rate (1 mg/kg/4 h vs. 6 mg/kg/24 h), the overall values of the 24-hour infusion studies tended to be higher than those of the 4-hour infusion studies (Table 2), indicating that 4-hour infusion was not sufficient to reach steady state. In particular, the brain penetration ($K_{p,uu,brain}$) of compound C and NFPS after 6 mg/kg/24 h infusion were 1.71- and 2.14-fold higher than the values after 1 mg/kg/4 h infusion, respectively (Table 2). When taking into consideration that $t_{1/2,terminal}$ values of compound C and NFPS were 6.8 and 4.59 hours (Table 1), the study design with 4-hour infusion could not be adequate to evaluate the brain penetration of a compound with a long half-life. In this study, most of the obtained $K_{p,uu,brain}$ and $K_{p,uu,CSF}$ for the commercial compounds were comparable with the previously reported values (Tables 2 and 3). However, the substantial difference between $K_{p,uu,brain}$ and $K_{p,uu,CSF}$ for loperamide and NFPS suggests that $K_{p,uu,CSF}$ or CSF concentration cannot be used as a surrogate marker reflecting unbound brain concentration of the compounds with poor brain penetration (Lin, 2008).

The modified study design with serial blood sampling during the infusion allowed the estimation of critical PK parameters of a compound, such as CL, V_{ss}, and $t_{1/2,eff}$, simultaneously in addition to the determination of $K_{p,uu}$. In the present study, we demonstrated that 4-hour infusion was not adequate to obtain accurate PK parameters, particularly for CL and V_{ss}, and 24-hour infusion is more accurate than 4-hour infusion on PK parameter determination. For CL, the average fold difference of the 4-hour infusion groups indicated that CL was overestimated by both NCA (2.1-fold) and compartment analyses (1.82-fold). In particular, the calculated CL of compound D after 4-hour infusion was 4.48-fold higher than the actual CL after intravenous bolus injection estimated by NCA, whereas a longer infusion time provided more accurate CL (1.26–1.40-fold; Table 4). For V_{ss}, the estimated values by both one- and two-compartment models from the 4-hour infusion studies tended to be underestimated, possibly due to the overestimated CL when considering the inverse association between CL and volume of distribution (Tables 4 and 5). For the same

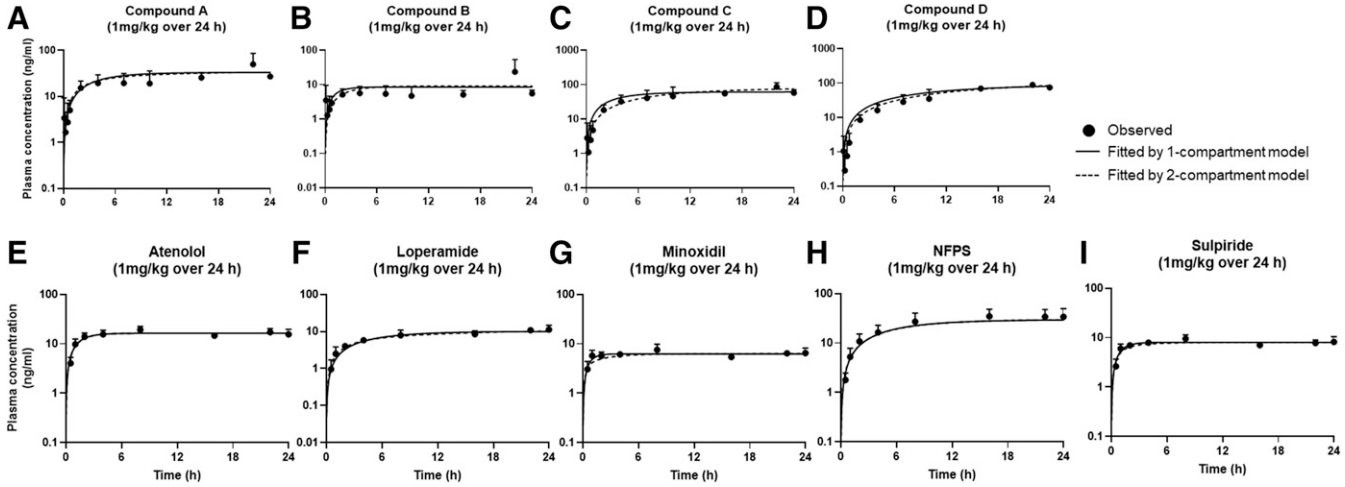


Fig. 3. Plasma concentration vs. time profiles of (A–D) internal and (E–I) commercial compounds in rats after a constant intravenous infusion over 24 hours as a cassette dosing (1 mg/kg). The closed circles represent observed data (n = 3; mean ± S.D.), and the fitted results by one- and two-compartment models are depicted as solid and dashed lines, respectively.

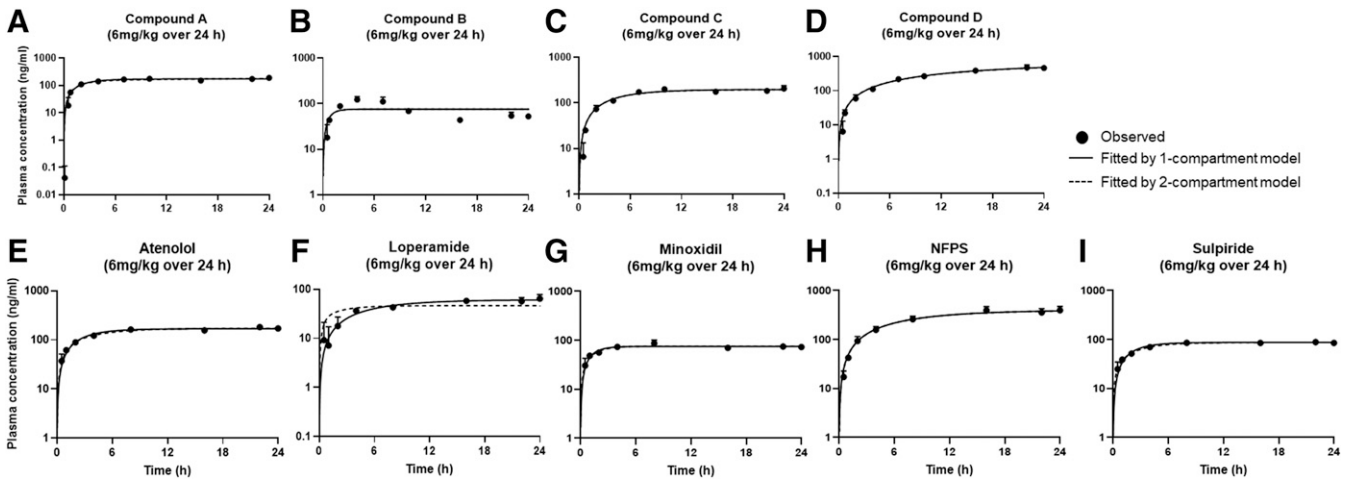


Fig. 4. Plasma concentration vs. time profiles of (A–D) internal and (E–I) commercial compounds in rats after a constant intravenous infusion over 24 hours as a cassette dosing (6 mg/kg). The closed circles represent observed data (n = 3; mean ± S.D.), and the fitted results by one- and two-compartment models are depicted as solid and dashed lines, respectively.

reason, studies with 24-hour, constant infusions exhibited better performance in estimating V_{ss} (Table 5). These results suggested that 24-hour infusion is a more appropriate study design for both $K_{p,uu,brain}$ and PK parameter estimation. Furthermore, the average fold differences indicated that the PK parameters from both 1 and 6 mg/kg/24 h infusion groups were similar and more accurate compared with the values of 1 mg/kg/4 h infusion groups (Tables 4–6), suggesting that infusion time is more critical for PK parameter estimation than infusion rate. We also demonstrated that compartment analysis is a useful approach to obtain PK parameters from a constant intravenous infusion study without elimination phase. Although the steady state of compound D was not achieved in the 4-hour infusion studies, compartment analyses using one- and two-compartment models provided more accurate CL values than NCA (Table 4). When one- and two-compartment models were applied to the 24-hour infusion studies (1 and 6 mg/kg), the average fold differences in CL and V_{ss} by one- and two-compartment models were similar with acceptable accuracy 1.07–1.42 and 1.13–1.29 for CL and 0.79–0.82 and 0.97–1.10 for V_{ss} (without loperamide), respectively (Tables 4 and 5). Similarly, $t_{1/2,eff}$ was also more accurately estimated by one-compartment model than two-compartment model when comparing average fold differences of 24-hour infusion studies in Table 6. Comparable performances of one- and two-compartment models in this study suggest that one-compartment model should be sufficient to obtain PK parameters from 24-hour constant infusion studies with no elimination phases in terms of model simplicity.

Infusion over 24 hours could be technically challenging sometimes depending on the physiochemical property of the compound (e.g., solubility of the compound). A formulation is generally needed that is stable over this time interval and physiologically acceptable for dosing in terms of volume and composition. Solubility (in aqueous solution) and stability of a compound are generally determined and optimized by chemist and formulation scientist prior to in vivo study, although it could still be challenging and not feasible to find the best combination of formulations for all the compounds. In this study, the best practice was applied, and dosing solutions were filtered prior to intravenous infusion. In addition, to avoid overestimated PK parameters caused by the compound's poor solubility, the actual drug concentrations of the dosing solution were also measured with the above-mentioned method and then used for further PK analyses.

As presented, we validated that both brain penetration and informative PK parameters of a compound could be successfully estimated by applying compartmental analysis to constant infusion studies. In drug development, the pharmacokinetics of a compound is generally determined in a separate study after testing brain penetration because of the nature of differences in the study designs. Very few studies have been reported in an effort to consolidate two different studies into one study. Bridges et al. (2014) developed a study design with single intravenous bolus dosing of compounds as a cassette dosing followed by another single intravenous bolus injection at 24 hours after the first dosing. The brain was then harvested at 15 minutes after the second dosing.

TABLE 6

$t_{1/2,eff}$ estimated by one- and two-compartment models based on 4- and 24-h intravenous infusion studies

Each value was normalized to the $t_{1/2,eff}$ of intravenous bolus data and then shown as avg. fold difference. The actual mean $t_{1/2,eff}$ values for each group were shown in the bracket (h).

	Internal Compounds				Commercial Compounds					Total	
	A	B	C	D	Atenolol	Loperamide	Minoxidil	NFPS	Sulpiride	Avg.	CV%
NCA											
Intravenous bolus	1 (1.16)	1 (5.65)	1 (2.25)	1 (15.1)	1 (1.83)	1 (1.43)	1 (0.79)	1 (4.44)	1 (1.18)	1	0
One-compartment analysis											
1 mg/kg over 4 h	1.95 (2.25)	0.16 (0.92)	2.44 (5.48)	0.15 (2.32)	0.23 (0.43)	0.18 (0.26)	0.32 (0.25)	0.26 (1.14)	0.29 (0.34)	0.66	132
1 mg/kg over 24 h	2.71 (3.13)	0.21 (1.21)	1.11 (2.49)	0.56 (8.45)	0.49 (0.89)	2.45 (3.51)	0.60 (0.47)	0.90 (3.99)	0.55 (0.65)	1.06	84.2
6 mg/kg over 24 h	1.27 (1.47)	0.09 (0.50)	1.27 (2.86)	0.93 (14.1)	1.01 (1.84)	2.90 (4.16)	1.01 (0.80)	1.21 (5.38)	1.18 (1.39)	1.21	60.5
Two-compartment analysis											
1 mg/kg over 4 h	2.93 (3.39)	0.51 (2.86)	3.00 (6.74)	0.11 (1.72)	0.42 (0.77)	0.18 (0.26)	1.44 (1.13)	0.30 (1.32)	0.40 (0.47)	1.03	113
1 mg/kg over 24 h	4.04 (4.67)	0.81 (4.55)	4.27 (9.61)	1.41 (21.2)	0.49 (0.90)	4.57 (6.56)	1.23 (0.96)	0.97 (4.33)	0.81 (0.95)	2.07	82.1
6 mg/kg over 24 h	2.80 (3.23)	0.32 (1.79)	1.70 (3.83)	4.92 (74.2)	1.25 (2.29)	0.75 (1.08)	1.11 (0.88)	1.21 (5.39)	1.37 (1.61)	1.71	80.6

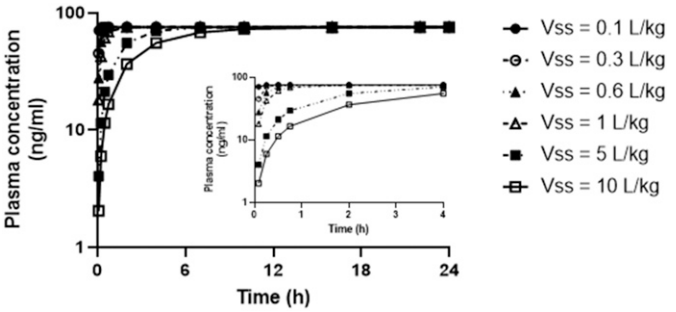


Fig. 5. Simulated plasma concentration vs. time profiles of compounds with low, moderate, and high volume of distribution after a constant intravenous infusion over 24 hours. The V_{ss} values were set to 0.1, 0.3, 0.6, 1, 5, and 10 l/kg, and the CL values were assumed to be the same as the hepatic blood flow rate (55 ml/min/kg). For simulation, it was assumed that the blood samples were serially collected at 0.083, 0.25, 0.5, 0.75, 1, 3, 5, 10, and 24 hours after infusion.

However, this method does not assure whether the steady state is achieved, leading to underestimation of $K_{p,uu,brain}$ for a compound, particularly with low permeability. Fu et al. (2018) established another study design with a single oral administration followed by intravenous infusion for 17 hours to evaluate bioavailability, $K_{p,uu,brain}$, and CL from one study. Although this approach allowed the evaluation of bioavailability with brain penetration of the target compound, V_{ss} could not be obtained from the study. Furthermore, both approaches by Bridges et al. (2014) and Fu et al. (2018) eventually had two different studies conducted sequentially while not consolidating two different studies into one study, since the brain penetration and the PK parameters of a compound were separately derived from two different studies that were performed in the same animals. In this study, we established a novel approach to evaluate brain penetration as well as critical PK parameters of the tested compounds by using compartmental analysis in one study with 24-hour constant infusion. According to the study by Jusko and Gibaldi (1972), about 90% of steady state could be achieved when a drug is infused for >3 mean residence time. Fu et al. (2018) reported that 88% of the compounds in the internal data base (>30,000 compounds) had a shorter mean residence time than 5 hours, inferring 24 hours of intravenous infusion used in the current study could be enough to reach steady state for most of the compounds in early drug discovery. Moreover, another strength of the 24-hour infusion study design is that it enables the assessment of V_{ss} from a constant intravenous infusion study with frequent sampling during infusion. Although a drug with low V_{ss} (<0.6 l/kg) (Smith et al., 2015) was not tested in this study, simulation proved that this study design is applicable to estimate V_{ss} of compounds with wide range of V_{ss} (Fig. 5).

However, the limitation of the current study is that the study design does not allow the estimation of bioavailability of test compound, and that is also an important aspect to be considered in drug development. Although an additional in vivo study is required to evaluate the bioavailability of compounds, the infusion study enables the narrowing down of compounds that could be further investigated. In other words, time and resources can be saved by performing bioavailability test for the optimal compounds with favorable brain penetration, CL, V_{ss}, and $t_{1/2}$. Further investigation is needed to develop a more efficient study design or PK approach for estimation of bioavailability along with other PK parameters (e.g., CL, V_{ss}, and $t_{1/2}$) as well as brain penetration.

In summary, we developed and validated a method to determine not only $K_{p,uu,brain}$ but also PK parameters from one single 24-hour intravenous infusion study design.

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Authorship Contributions

Participated in research design: Liu, Wei.
Conducted experiments: Noh, Pietrasiewicz.
Performed data analysis: Noh.
Wrote or contributed to the writing of the manuscript: Noh, Liu, Wei.

Note Added in Proof—Some values of Sulpiride were not correctly cited in Tables 1 and 2 in the Fast Forward version published December 1, 2020. Tables 1 and 2 have now been corrected.

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